Supplemental Data for

Tie2 protects the vasculature against thrombus formation in systemic inflammation

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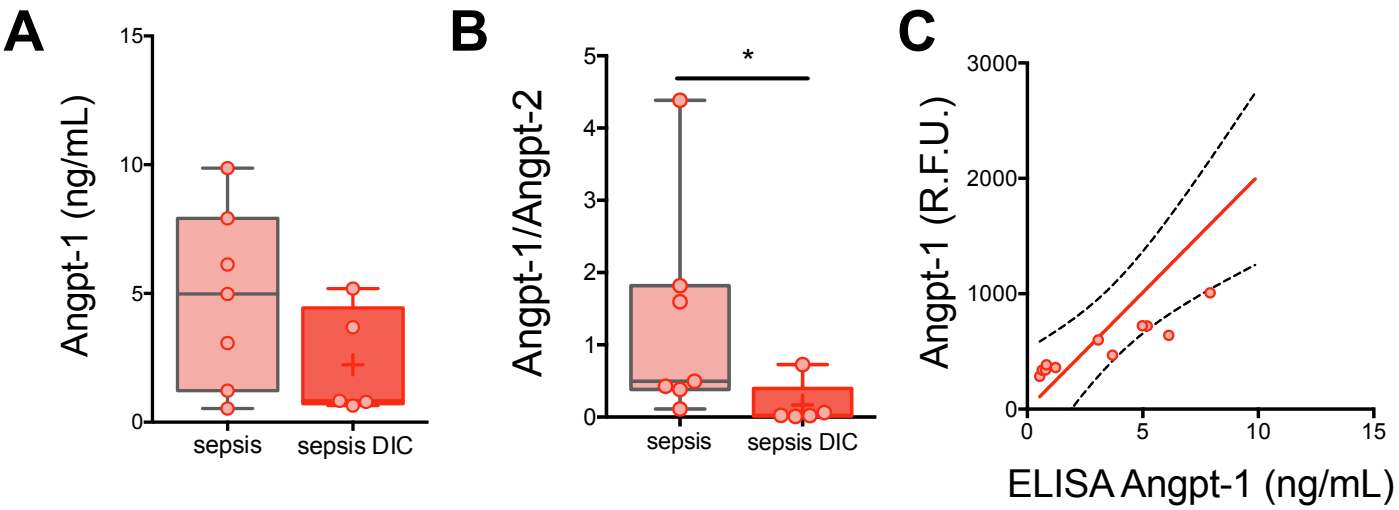
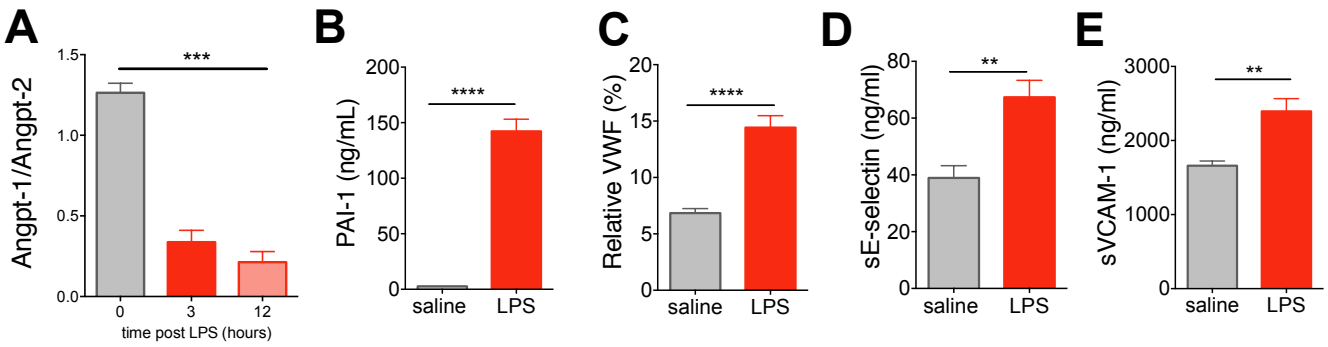
Characteristic	Sepsis (*n*=7)	Sepsis DIC (*n*=7)	*P* value
Age (year)	62 (39.2 – 65.7)	60 (41.0 – 72.0)	0.6958
Female	2 (25%)	3 (37.5%)	*ns* (1.0)
Race, *n* (%)			
Asian/Pacific Islander	0	2 (28.6%)	
Caucasian	7 (100%)	4 (57.1%)	*ns* (0.2)
Hispanic	0	1 (14.2%)	
min platelet count	186.0 (152.0 - 286.0)	21.0 (14.0 – 49.0)	0.0006

Supplemental Table 1. Demographics and clinical characteristics of the sepsis and sepsis DIC study population.

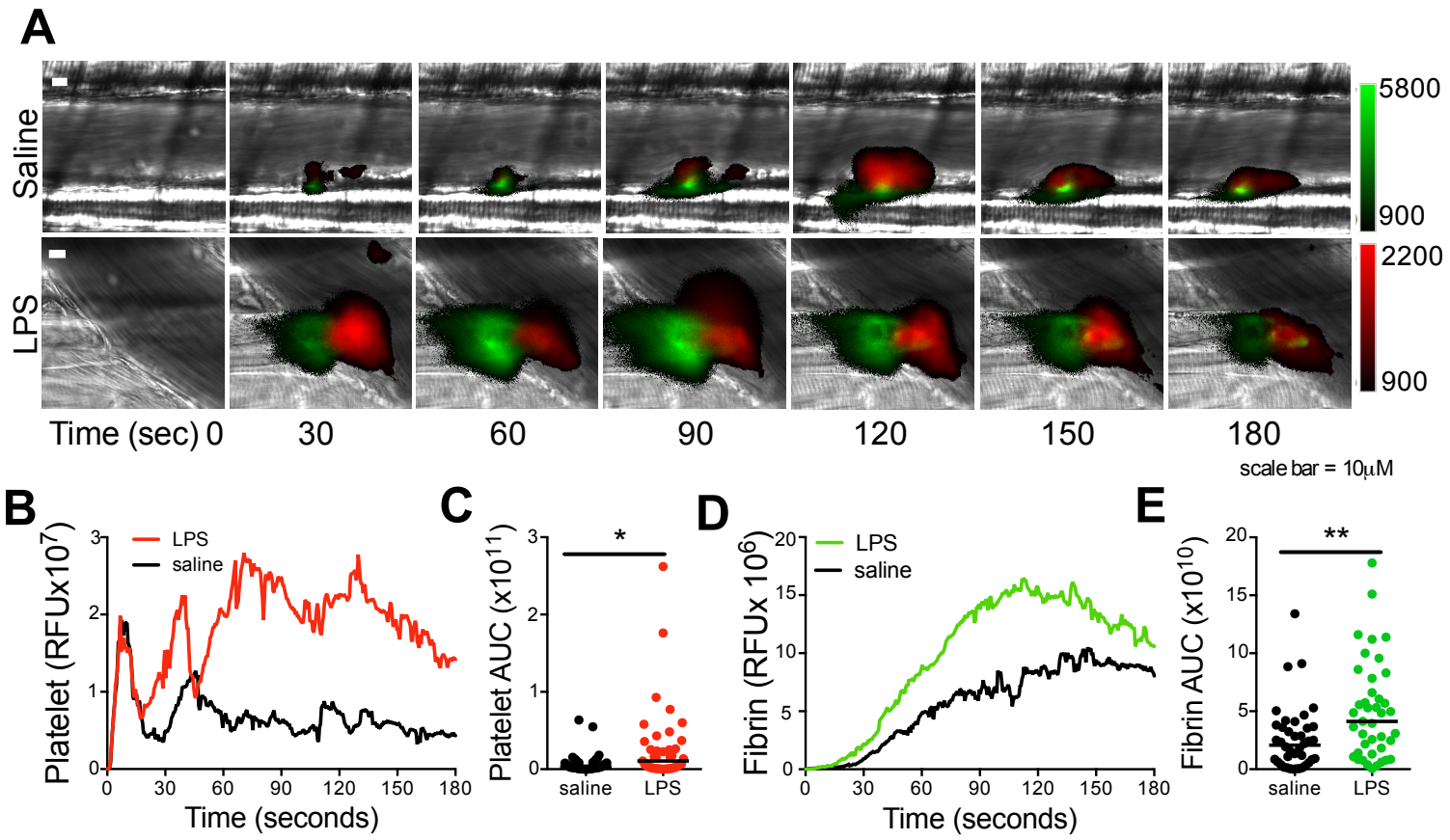


Supplemental Figure 1. Plasma angiopoietin levels in sepsis DIC discovery cohort.
(**A**) Plasma Angpt-1 levels determined by ELISA, n = 5 for Angpt-1 sepsis DIC plasma and ratio measurements due to insufficient volume for n = 2 samples for ELISA analysis. **(B)** Within-patient relative Angpt-1 to Angpt-2 levels measured by ELISA. *P<0.05, **P<0.005, Mann-Whitney U test. **(C)** Scatter plot showing correlation between the two methods of analysis (SOMAScan and ELISA) for plasma Angpt-1 levels (r^2 = 0.6303, P = 0.0020, n = 12).

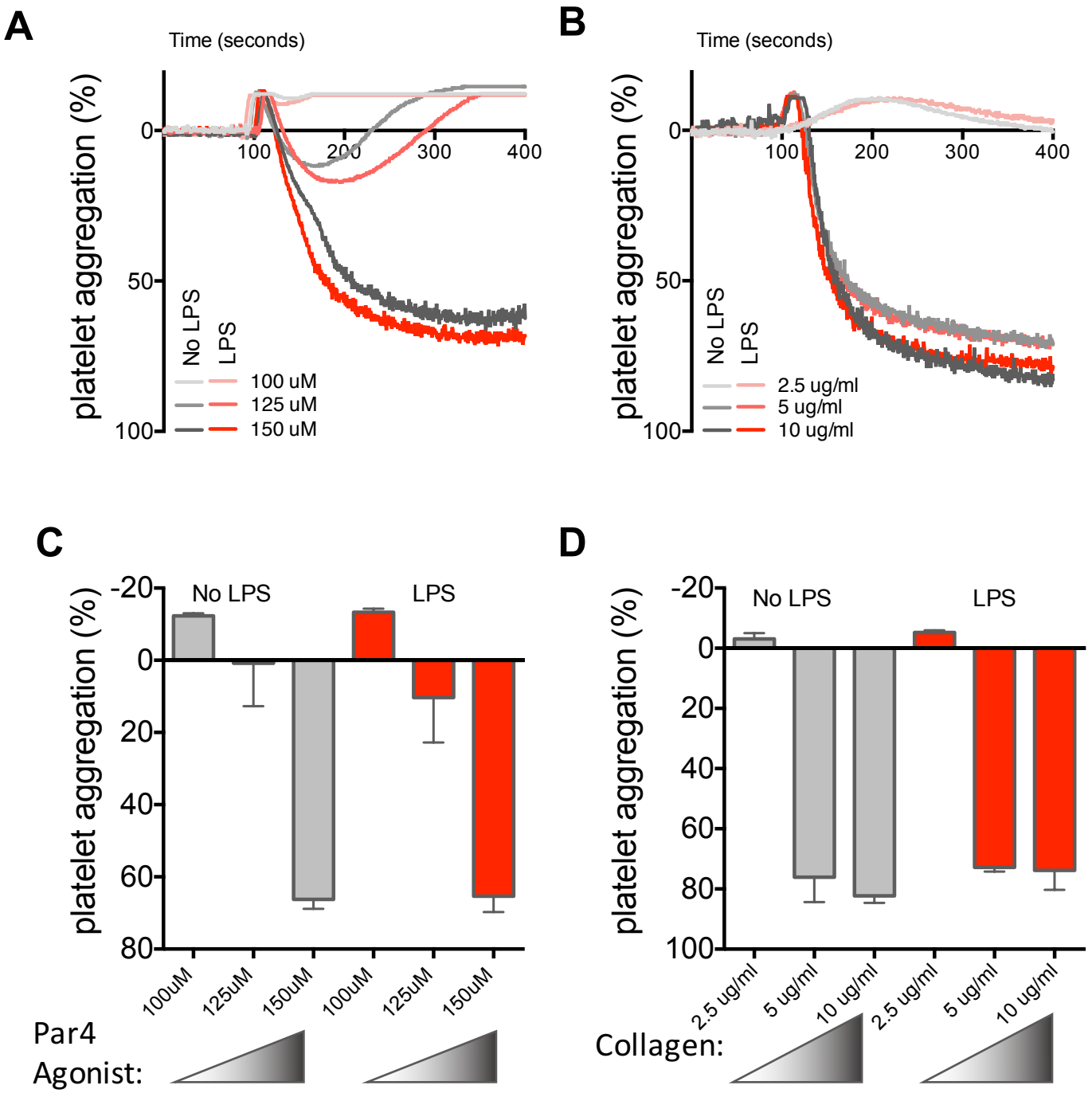


Supplemental Figure 2. Plasma angiopoietin levels and markers of endothelial activation in a murine LPS-induced DIC model.

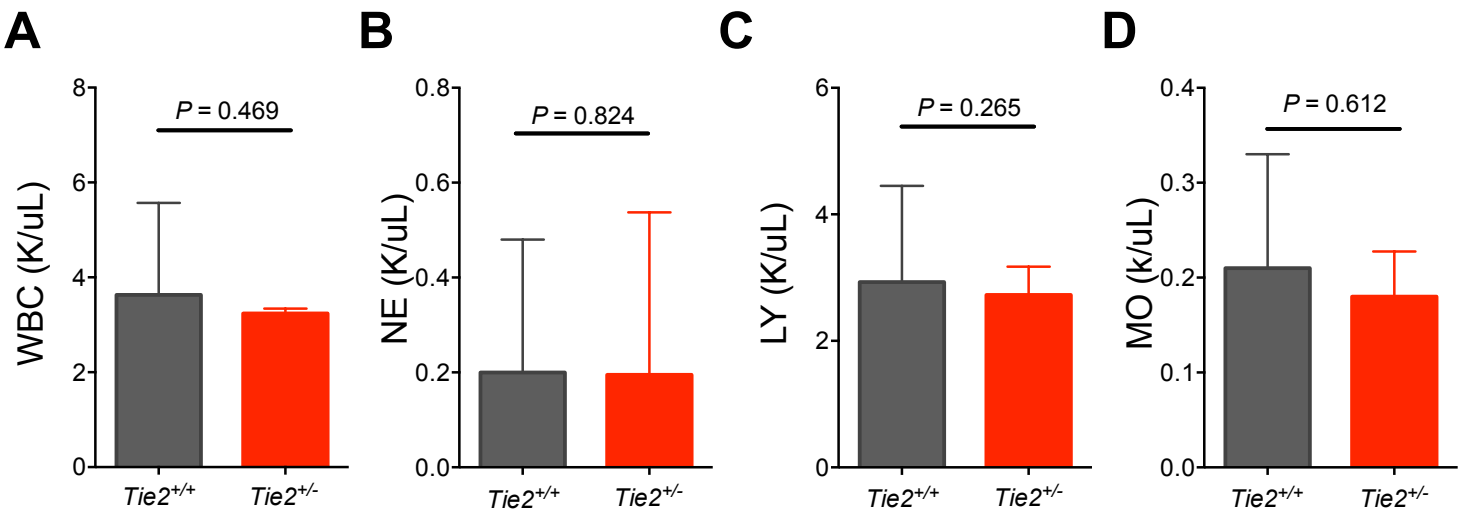
(A) Relative plasma Angpt-1 to Angpt-2 ratio kinetics in in C57Bl/6J mice challenged with LPS (10 mg/kg) or saline measured at specific time points (0, 3 and 12 hours). ***P = 0.0002, Kruskal-Wallis ANOVA test. Markers of endothelial activation, including (K) Plasminogen activator inhibitor-1; PAI-1, (L) Von Willebrand factor; VWF, (M) soluble E-selectin and (N) soluble VCAM-1 measured by ELISA in plasma of C57Bl/6J mice challenged with LPS for 3 hours or saline. Data represent mean +/- SEM ($n \geq 5$ per group). ** $P < 0.01$, *** $P < 0.001$, Mann Whitney U.



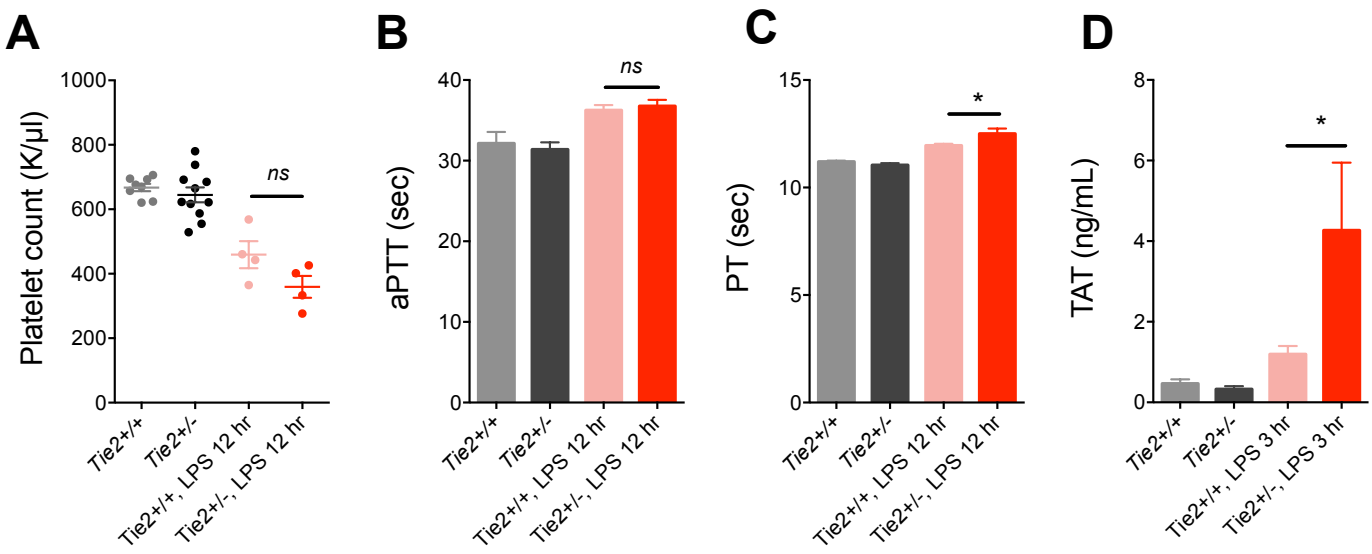
Supplemental Figure 3. Prothrombotic response in venules following LPS.
Platelet and fibrin accumulation at sites of laser injury was monitored in cremaster venules of C57Bl/6J mice 1-3 hours following saline or 10 mg/kg LPS injection. Platelets and fibrin were visualized by infusion of anti-platelet (CD42b; 0.1 mg/g body weight) and anti-fibrin (59D8: 0.5 mg/g body weight) antibodies, conjugated to Dylight 649 and 488 respectively. (**A**) Representative binarized images of a thrombus (*red = platelets; green = fibrin*) for saline and LPS-treated mice. Median integrated fluorescent intensities and area under the curve (AUC) for individual thrombi were calculated for platelets (**B,C**) and fibrin (**D,E**) in saline (*black*) and LPS-treated mice (*red or green*). (**c,e**) Data are represented as median AUC of individual thrombi (saline *n* = 40; LPS *n* = 41), *P<0.05; **P<0.01, Mann Whitney *U* test.



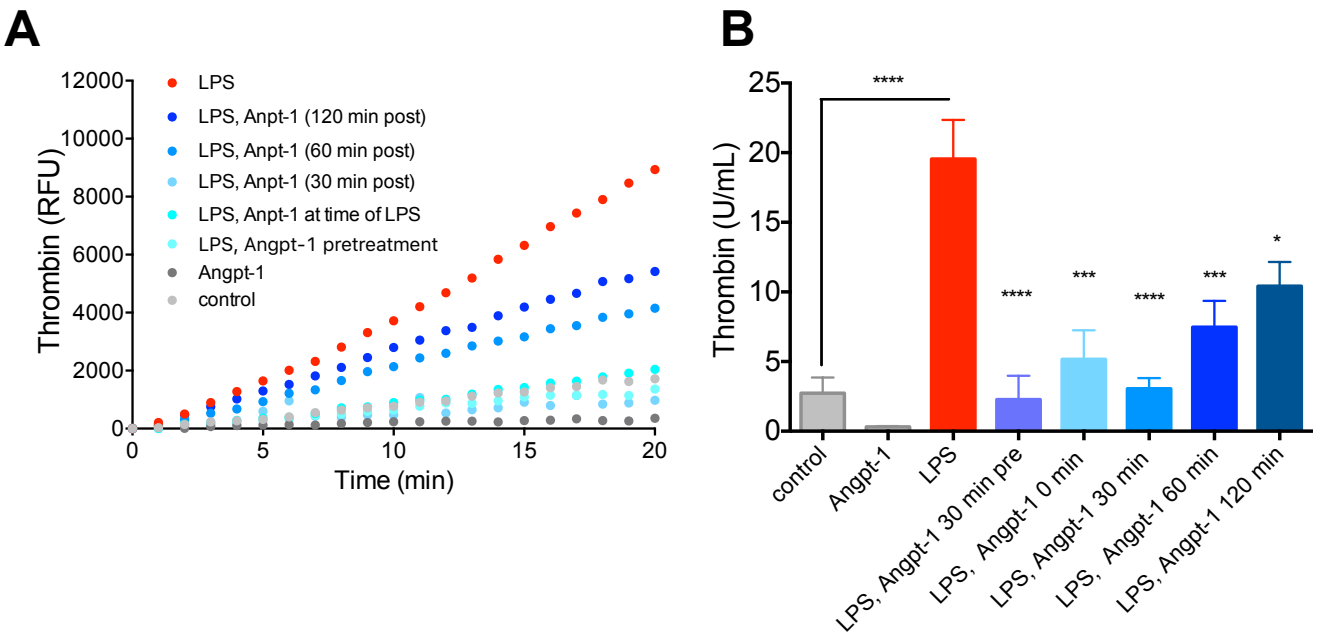
Supplemental Figure 4. Platelet aggregation sensitivity to agonists following LPS.
(A) PAR4- and **(B)** collagen induced platelet aggregation was measured in platelet rich plasma obtained from C57Bl/6J mice with no LPS or with 3 hours of LPS exposure showing representative aggregation experiment of 2 independent experiments (LPS = red tracing; No LPS = grey tracing). Quantification of PAR4- **(C)** and collagen **(D)** induced aggregation at 250 sec after sub-threshold and escalating doses of agonist, as indicated. Data represent mean ± SEM (*n* = 3 – 7 replicates per dose).



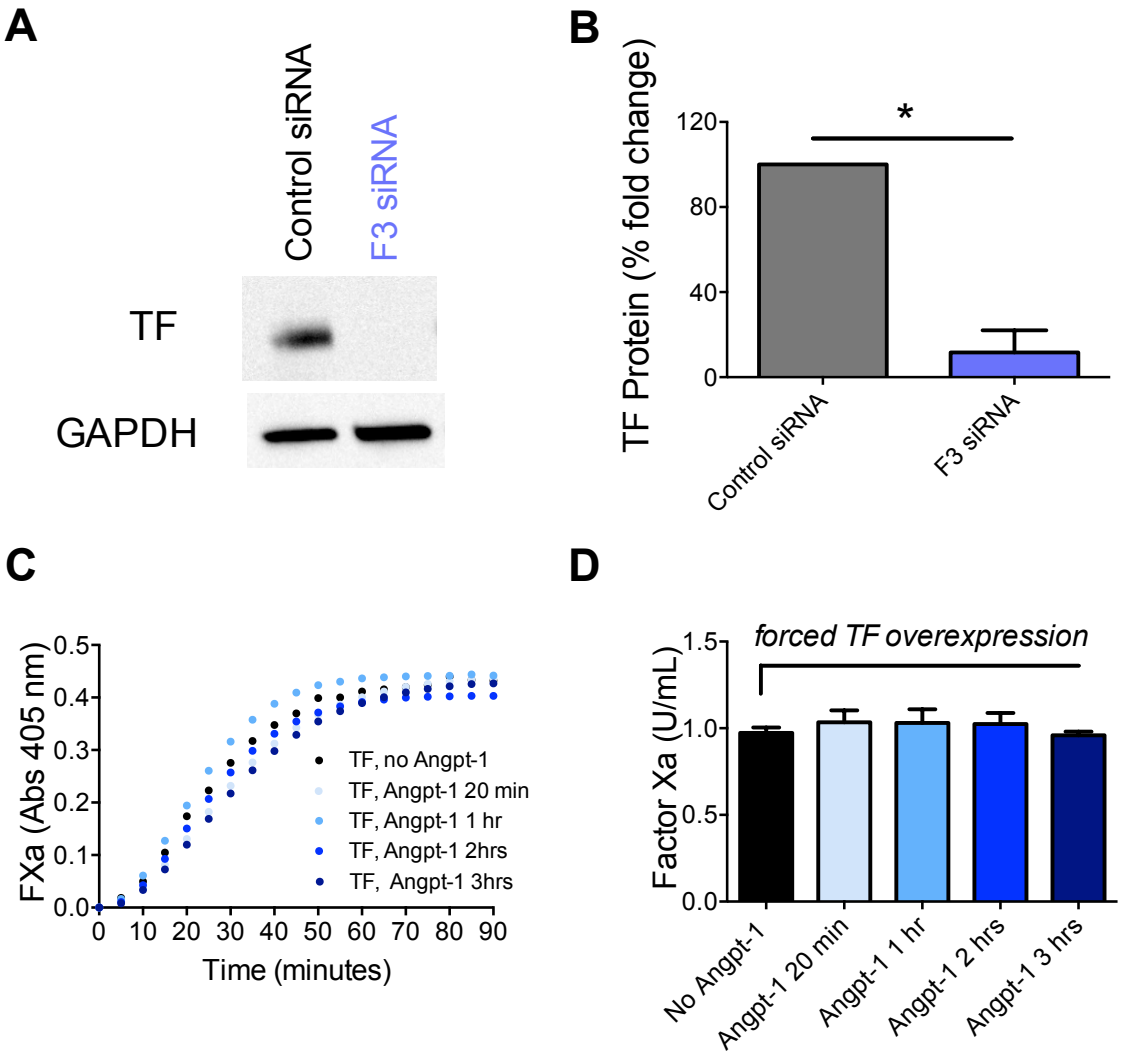
Supplemental Figure 5. Systemic measures of blood parameters in Tie2 heterozygous (*Tie2*$^{+/-}$) and wild-type littermate controls (*Tie2*$^{+/+}$).
Complete blood count in mice with indicated genetic background. **(A)** WBC, white blood cells; **(B)** NE, neutrophils; **(C)** LY, lymphocytes; **(D)** MO, monocytes. *P<0.05, **P<0.01, one-way ANOVA, *n* = 7 – 10 per group.



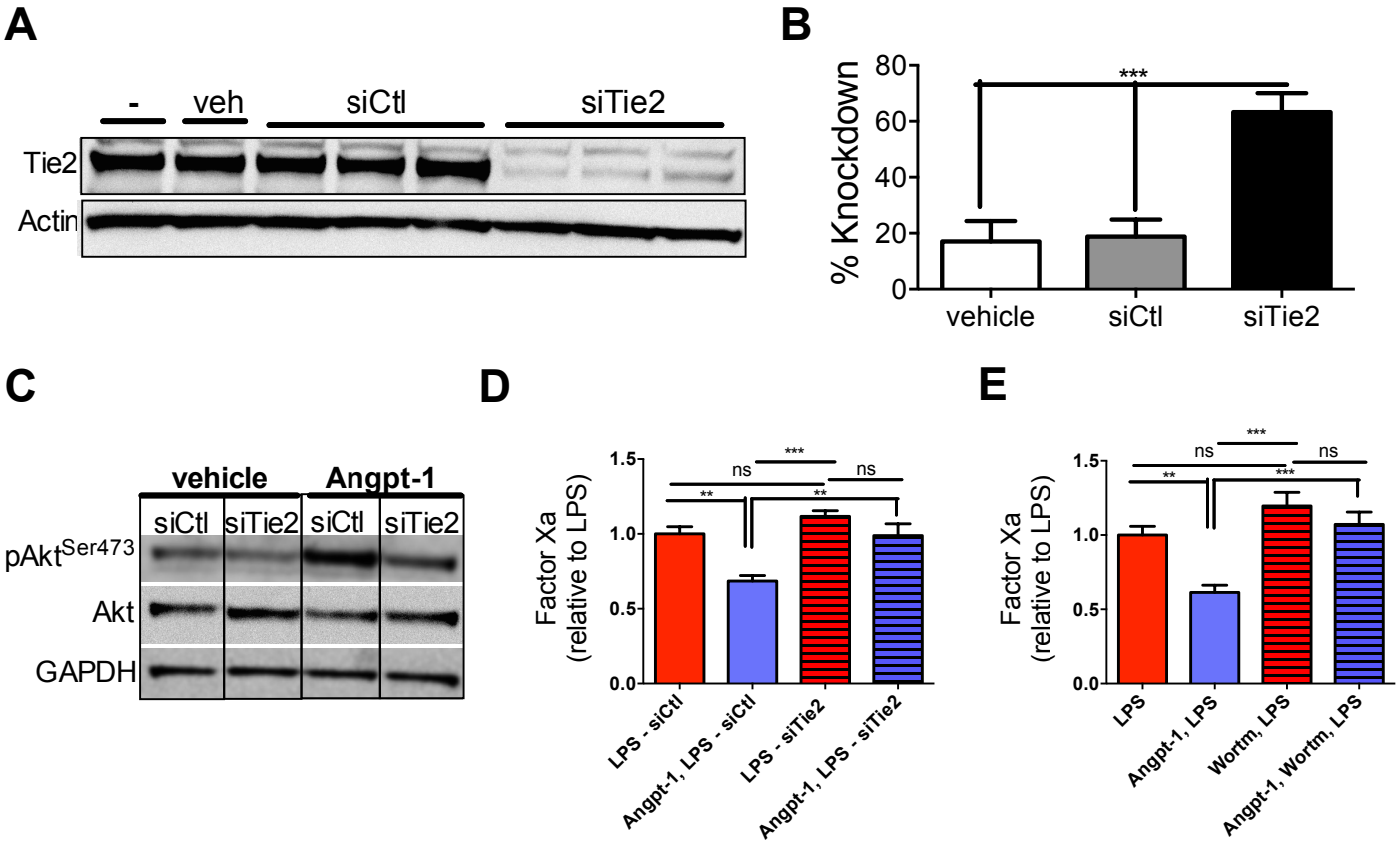
Supplemental Figure 6. Exacerbated coagulation parameters and markers of thrombin generation in Tie2 heterozygous (*Tie2*$^{+/-}$) mice with LPS.
(A) Whole blood platelet count, **(B)** aPTT (activated partial thromboplastin time), **(C)** PT (pro-thrombin time) and **(D)** levels of circulating thrombin-anti-thrombin (TAT) complex measured in CD-1 Tie2 heterozygous mice (*Tie2*$^{+/-}$) and wild-type littermate controls (*Tie2*$^{+/+}$) mice with samples collected at baseline (no LPS) and the indicated times post challenge with LPS (12.5 mg/kg). *P < 0.05, **P < 0.01, one-way ANOVA with post test comparing differences between genotype at baseline and post LPS.



Supplemental Figure 7. Angiopoietin-1 application post LPS stimulation suppresses prothrombotic actions on the endothelium.
Thrombin generation was determined on human umbilical vein endothelial cells (HUVECs) incubated with Angpt-1 (200 ng/ml) at the indicated time per and post exposure of HUVECs to LPS complex. Representative experiment is depicted as **(A)** arbitrary fluorescent units (RFU) as a function of time and the **(B)** rate of the reaction for thrombin generation converted to units/ml and normalized to controls. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, one-way ANOVA with Bonferroni's post-test.

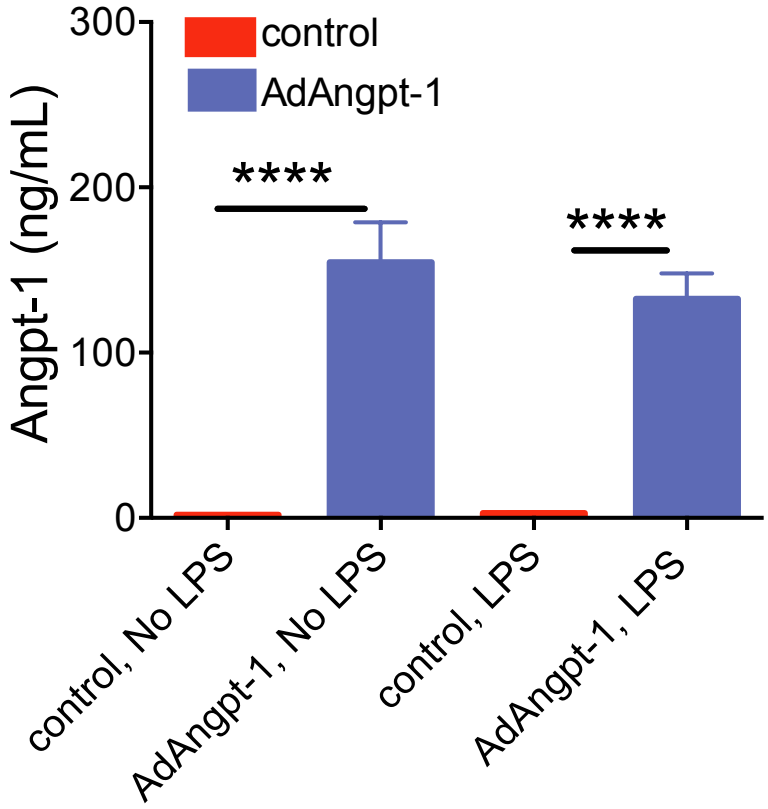


Supplemental Figure 8. Tissue factor expression in endothelial cells.
(A) To evaluate the specificity of the tissue factor antibody, HUVECS exposed to either control siRNA or siRNA directed at tissue factor. HUVECs were then stimulated with TNF-α (10 ng/ml; Millipore) for 3.5 hr and evaluated for tissue factor expression by Western blot analysis. A representative western blot is shown. (**B**) Quantification of tissue factor expression using densitometry (*N* = 2 representative experiments). (**C,D**) Ea.hy926 cell line stably expressing tissue factor was incubated with control or Angpt-1 (200 ng/ml), independent of LPS, for indicated durations and FXa generation was measured using chromogenic substrate. (**C**) Absorbance at 405 nm is depicted as a function of time. (**D**) The rate of the reaction was converted to units/ml and normalized to controls. Data represent mean ± SEM (*n* = 3) from a representative experiment.

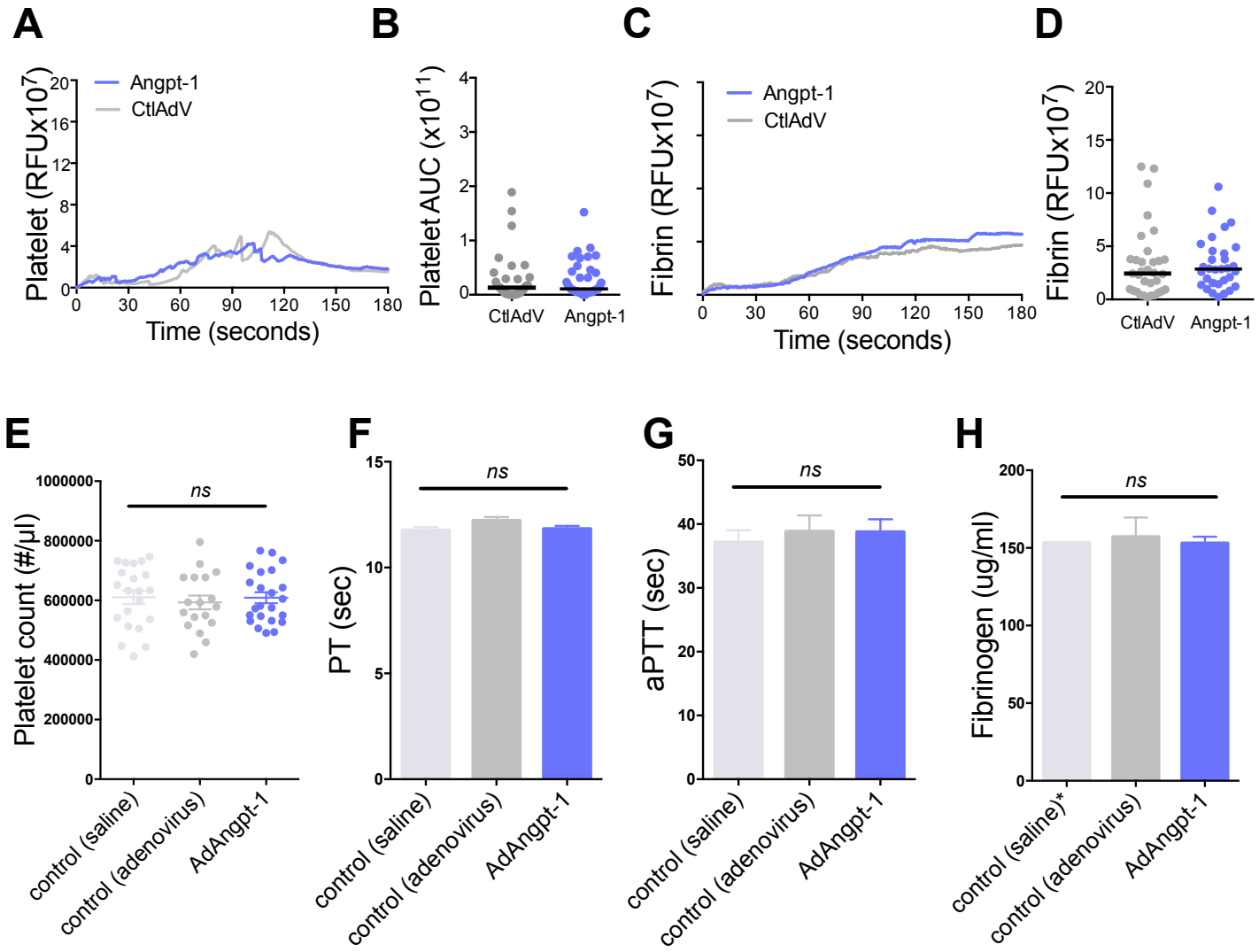


Supplemental Figure 9. Angiopoietin-1 blunts LPS-induced Factor Xa generation on endothelial cells in a Tie2-PI3K dependent fashion.

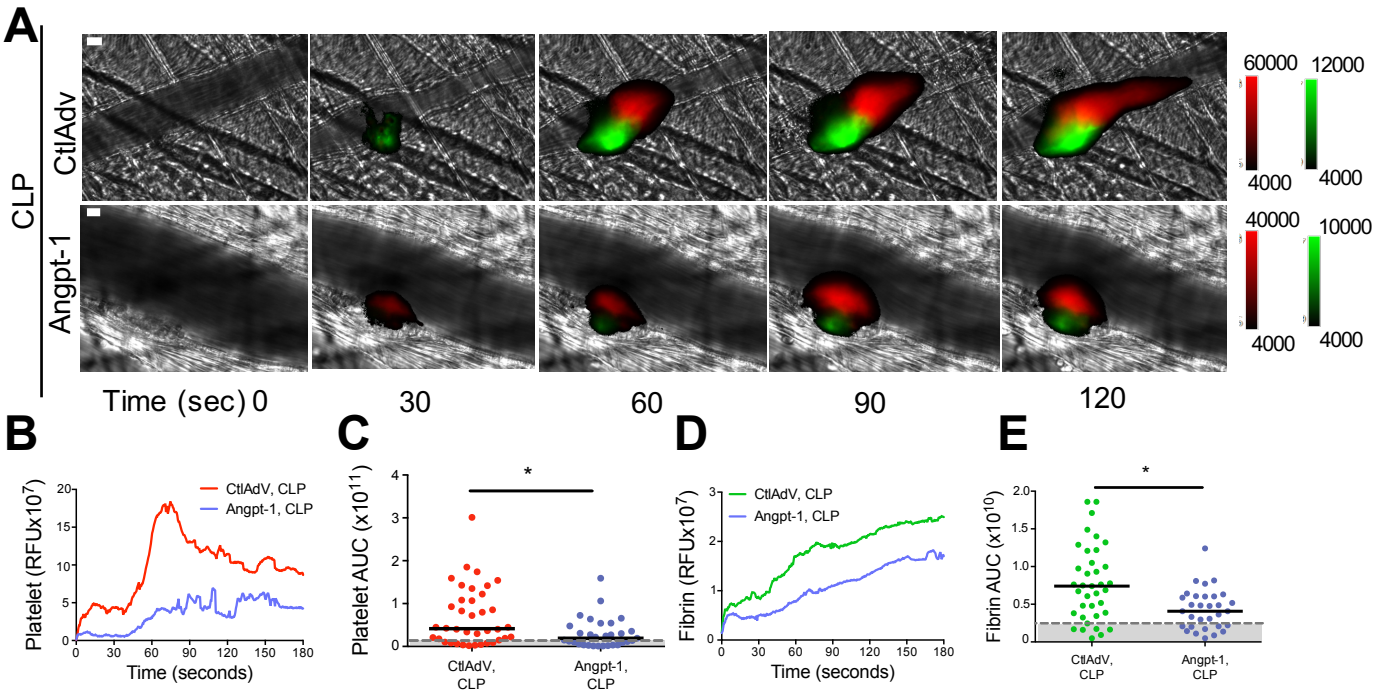
(**A-D**) HUVECs were transfected with control siRNA (siCtl) or Tie2 siRNA (siTie2) for 48 hours. (**A**) Immunoblot of Tie2 knockdown. (**B**) qRT-PCR data of 3 independent experiments, ***P<0.001, one-way ANOVA with Bonferroni's post-test. (**C**) siRNA-treated HUVECs were incubated with vehicle or Angpt-1 (200 ng/ml) for 30 min. Immunoblot analysis of HUVEC lysates for pAkt^{Ser473}, total Akt and GAPDH. (**D**) siRNA-treated HUVECs were pre-incubated with vehicle or Angpt-1 (200 ng/ml) for 30 min prior to addition of LPS complex. FXa generation was measured using a chromogenic substrate (Abs 405 nm). The rate of the reaction was converted to units/ml and normalized to LPS. Data represent mean ± SEM, *n* = 6 from two independent experiments. **P <0.01,***P <0.001, one-way ANOVA with Bonferroni's post-test. (**E**) HUVECs were pre-incubated with vehicle, Angpt-1 (200 ng/ml) and/or PI3K inhibitor wortmannin (100 nM) for 30 min prior to addition of LPS complex. FXa generation was measured using a chromogenic substrate (Abs 405 nm). The rate of the reaction was converted to units/ml and normalized to LPS. Data represent mean ± SEM (*n* = 3). *P<0.05, **P<0.01, one-way ANOVA with Bonferroni's post-test.



Supplemental Figure 10. **Angiopoietin-1 levels in experimental adenoviral mice.**
Angpt-1 levels in plasma of C57Bl/6J mice injected adenovirus expressing Angpt-1 (AdAngpt-1) or a control adenovirus (control) with and without LPS (10 mg/kg).

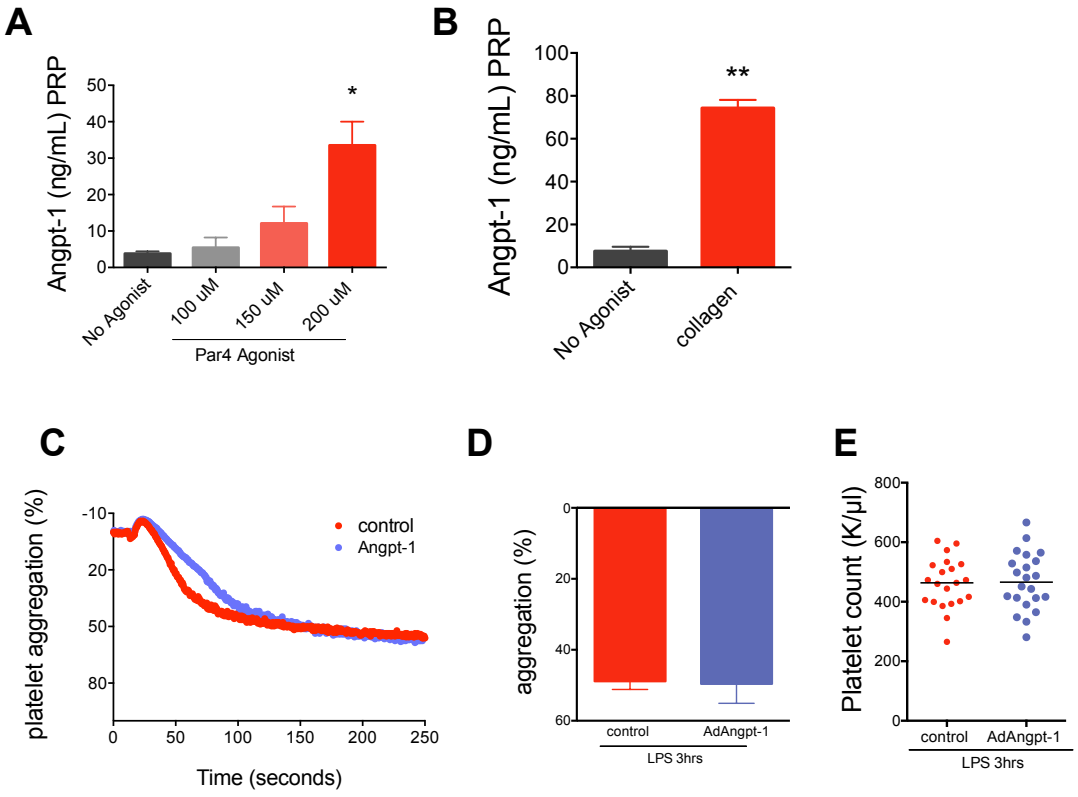


Supplemental Figure 11. Baseline thrombus formation and coagulation parameters in experimental mice. (**A-D**) Representative binarized images of thrombus formation following laser injury in mice injected with control adenovirus (CtlAdv) (top) or an adenovirus expressing Angpt-1 (AdAngpt-1). Median integrated platelet (**A,B**) and fibrin (**C,D**) fluorescent intensities (**A,C**) and AUC (**B**,**D**) were calculated for individual thrombi from representative binarized images of thrombus formation following laser injury in mice injected with control adenovirus (CtlAdv; grey) or an adenovirus expressing Angpt-1 (AdAngpt-1; blue). CtlAdv (n = 2; 33 thrombi), AdAngpt-1 (n = 3; 38 thrombi). **(E)** Whole blood platelet count, (**F**) PT (pro-thrombin time), (**G**) aPTT (activated partial thromboplastin time), and (**H**) plasma fibrinogen levels were measured in C57Bl/6J mice treated with CtlAdV, AdAngpt-1 or vehicle saline.

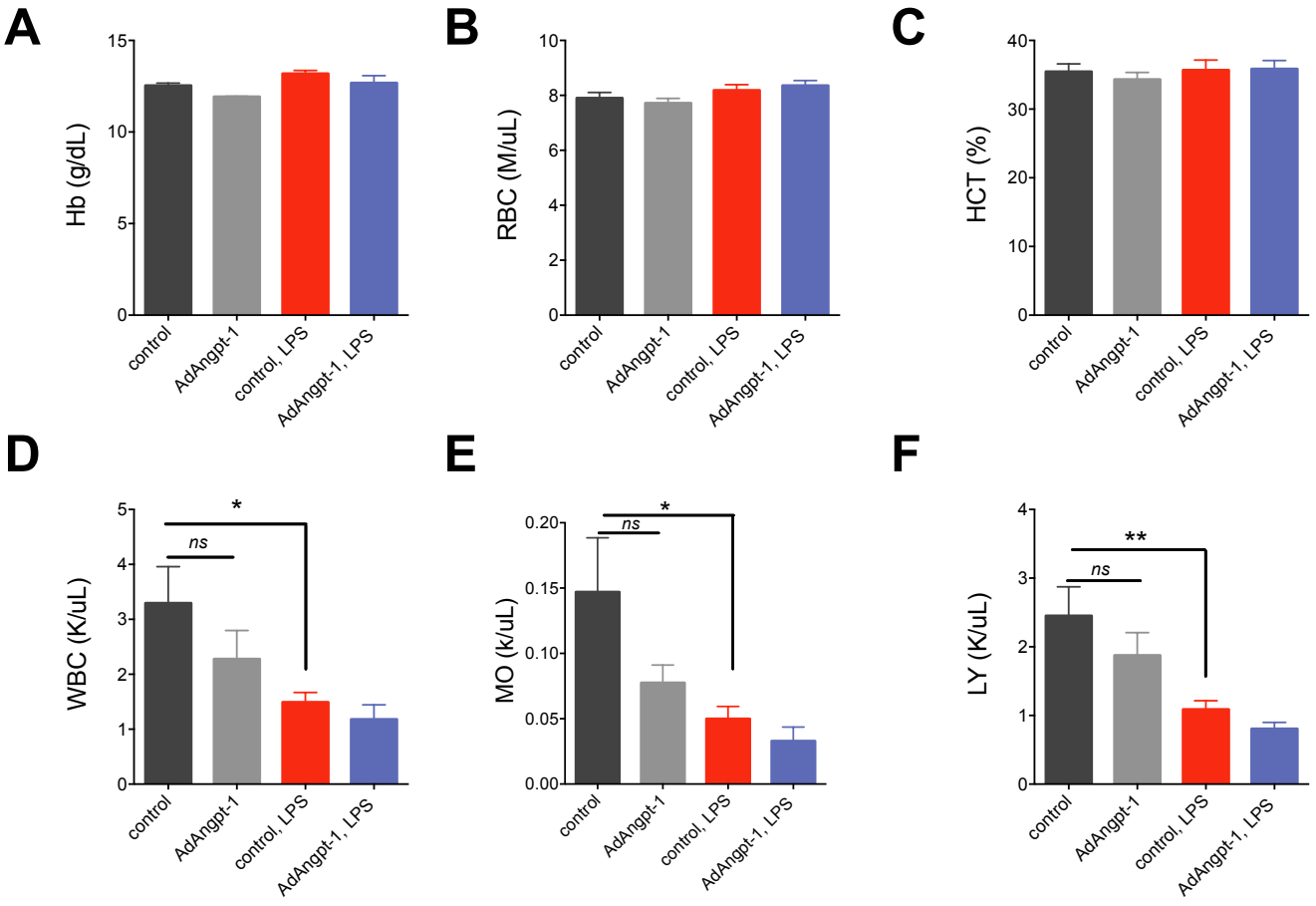


Supplemental Figure 12. Tie2 activation normalizes the injury-induced thrombotic response in a cecal ligation and puncture (CLP) polymicrobial sepsis model.

Representative binarized images of thrombus formation (**A**) following laser injury in mice injected with control adenovirus (CtlAdv) (top) or an adenovirus expressing Angpt-1 (AdAngpt-1) post cecal ligation and puncture (1-3 hrs) (*platelets = red; fibrin = green*). Median integrated platelet (**B**) and fibrin (**D**) fluorescent intensities and AUC (**C, E**) were calculated for individual thrombi, CtlAdv plus CLP (*n* = 3; 38 thrombi), Angpt-1 plus CLP (*n* = 3; 31 thrombi).



Supplemental Figure 13. **Angiopoietin-1 release and *ex vivo* platelet aggregation.**
Angpt-1 levels in platelet rich plasma (PRP) following incubation with PAR-4 agonist (**A**) or collagen (**B**). Data represents mean +/- SEM (*n* = 5 for Par4 agonist experiment; *n* = 4 for collagen experiment). *:*P* <0.05, one-way ANOVA; **: *P* = 0.02, Mann Whitney *U*. **(C)** Representative platelet aggregation measured in PRP of mice injected with control adenovirus or AdAngpt-1 (*control: red tracing; Angpt-1=blue tracing*). **(D)** Quantification of PAR-4-mediated aggregation at 250 sec. **(E)** Platelet counts collected 3 hrs post LPS. Data represent mean ± SEM (*n* = 4 - 6). *P<0.05, **P<0.01, one-way ANOVA, *n* = 5 from one representative experiment.



Supplemental Figure 14. **Systemic measures of blood parameters in mice with adenoviral intervention.** Complete blood count at 3 hours post LPS or saline in mice treated with AdAngpt-1 or a control adenovirus. **(A)** RBC, red blood cells; **(B)** Hb, hemoglobin; **(C)** HCT, hematocrit; **(D)** WBC, white blood cells; **(E)** MO, monocytes; **(F)** LY, lymphocytes. *P<0.05, **P<0.01, one-way ANOVA, *n* = 5 from one representative experiment.